

EpiRILs

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Running title: EpiRILs: lessons from Arabidopsis

EpiRILs: lessons from Arabidopsis

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1 **Abstract**

2 In recent times, epigenetic marks have emerged as important players involved in the
3 regulation of gene expression and transposable element silencing in many organisms. In
4 plants, many epigenetic changes, mainly at the level of DNA methylation, are
5 transgenerational stable and contribute to formation of epialleles, affecting developmental
6 and agronomical traits. In this scenario, it becomes critical to differentiate the genetic from
7 the epigenetic contribution to plant phenotypes. In Arabidopsis, epigenetic Recombinant
8 Inbred Lines (epiRILs), obtained by an initial cross of isogenic parents with different DNA
9 methylation profiles, provide a powerful tool to investigate the role and significance of
10 epigenetic alteration in identical or almost identical genetic backgrounds. Such populations
11 have greatly increased our knowledge in mechanisms involved in epialleles formation and
12 stability, as well as in the consequences of DNA methylation changes in genomic stability,
13 transposable elements activation and phenotypic traits.

14

Introduction

While it is known that DNA is the support of heredity, it is more and more recognised that heritable phenotypic variation can be also caused by epigenetic changes, and not only by change in the DNA sequence. Methylation of cytosines (DNA methylation) is an epigenetic mark conserved across many species and plays an important role in regulating gene expression (X. J. He, Chen, & Zhu, 2011). Widespread perturbation in DNA methylation has been shown to lead to heritable phenotypic changes in plants (X. J. He et al., 2011; Seymour & Becker, 2017). Moreover, in plants changes in DNA methylation can be transmitted through generations because, contrary to what occurs in mammals, there is no clear evidence of global DNA methylation resetting at each generation (Heard & Martienssen, 2014). DNA methylation in plants occurs at cytosines and can be observed in all three contexts CG, CHG and CHH. DNA methylation in genes is almost only observed in the CG context, and seems to be associated with gene activation. On the other hand, dense DNA methylation at Transposable Elements (TEs) is observed in all three contexts and is associated with transcriptional repression (Gehring & Henikoff, 2007).

To date only a handful of natural epialleles (see definitions) have been described in eukaryotes (Table 1). These epialleles are characterised by a gain or loss of DNA methylation, often associated with a change in gene expression, as well as strong phenotypes. Such changes of DNA methylation at epialleles are usually observed in repeated sequences or TEs that are either in close proximity or inside genes. While these epialleles are stable over a generation, some are metastable, which means that some level of instability as well as gradual reversions have been observed. This is in striking contrast to

genetic alleles (see definitions), as changes in the DNA sequence are more stable than changes in DNA methylation for several orders of magnitude (Becker et al., 2011). The small number of natural epialleles described so far could be explained by the fact they were either identified thanks to a strong phenotypes (Bender & Fink, 1995; Cubas, Vincent, & Coen, 1999; Manning et al., 2006; Martin et al., 2009; Melquist, Luff, & Bender, 1999; K. Miura et al., 2009; L. Zhang et al., 2012; X. Zhang, Sun, Cao, & Song, 2015), to an allelic incompatibility between accessions (Agorio et al., 2017; Blevins, Wang, Pflieger, Pontvianne, & Pikaard, 2017; Durand, Bouche, Perez Strand, Loudet, & Camilleri, 2012) or by chance (Silveira et al., 2013). We can suppose that many epialleles have not been discovered yet as they might be associated with mild phenotypes, or phenotypes only visible under certain circumstances (e.g environmental stress). Moreover, while identifying alleles underlying a certain phenotype is nowadays straight-forward, identifying DNA methylation changes at an unknown position associated with a phenotype is still challenging and requires more sophisticated analysis. In the context of this chapter, we will use a broad definition of epialleles: any stably transmitted change in methylated profiles, with or without phenotypic consequences.

[Insert Table 1 here]

Perturbing DNA methylation, by mutating genes involved in DNA methylation deposition or maintenance, is a way to increase the chance of detecting epialleles. Hence epialleles have been detected in *Arabidopsis thaliana* mutants characterised with a global loss of DNA methylation. Some of these epialleles are characterised by DNA hypomethylation, such as *fwa*, associated with a late flowering phenotype (Kakutani, 1997; Kakutani, Jeddeloh, Flowers, Munakata, & Richards, 1996; Lippman et al., 2004; Ronemus, Galbiati, Ticknor, Chen, & Dellaporta, 1996; Soppe et al., 2000) and *sqn*, associated with an increased

expression (Catoni et al., 2017; Habu et al., 2006). Others are characterised by DNA hypermethylation, such as *sup*, associated with an excess of stamens (Jacobsen & Meyerowitz, 1997; Jacobsen, Sakai, Finnegan, Cao, & Meyerowitz, 2000), *ag*, associated with an absence of carpels (Jacobsen et al., 2000) and *bns*, associated with a dwarf phenotype (Saze & Kakutani, 2007). These epialleles are stably maintained after removal of the inducible mutation, with a certain degree of metastability, as also observed for natural epialleles. Except for QQS (Silveira et al., 2013), until now, none of these induced epialleles have been naturally observed in *Arabidopsis thaliana*.

In order to identify alleles with milder or quantitative phenotypes (in contrast to strong qualitative phenotypes), recombinant inbred lines (RILs, see definitions) are commonly used (Mackay, 2001). These populations are used to identify loci at which the segregation of parental alleles are associated with phenotypic changes. Such an approach could also allow the detection of epialleles associated with mild or quantitative phenotypes. However, as this will be described in more detail in this chapter, alleles as well as epialleles are segregating in RIL populations, making it difficult to separate epigenetic from genetic impact on phenotypes (Johannes, Colot, & Jansen, 2008). In order to specifically identify epialleles associated with phenotypic changes, epigenetic RIL (epiRILs, see definitions) have been generated in *Arabidopsis thaliana* (Johannes et al., 2009; Reinders et al., 2009). In short these populations have been created in order to maximise DNA methylation changes, while reducing (if not completely removing) DNA sequence differences.

In this chapter we will be discussing the many aspects in which epiRIL populations have been of a great use and how the acquired knowledge could be translated in crops in the future.

86 -----

87 Definitions

88 Allele: Genetic variants of a gene. Different alleles can result in different phenotypic traits.

89 Epiallele: Epigenetic variants of a gene. The genetic sequence of the epialleles is identical,
90 but the level of DNA methylation, or other epigenetic marks, are different. Epialleles can
91 result in differences in gene expression, which can potentially lead to differences in
92 phenotypic traits.

93 RIL (Recombinant Inbred Lines): Set of homozygous lines that incorporate a combination of
94 genomic regions derived from the cross of two parent lines. Each RIL is developed by self-
95 pollination and single seed descent propagation of a segregating F2 plant obtained from the
96 initial cross. Inbreeding continues for at least six/eight generations, determining the fixation
97 in homozygous form of most of alleles and epialleles. RILs are often used for mapping
98 QTLs.

99 EpiRIL (Epigenetic Recombinant Inbred Lines): Similarly to RILs, epiRILs are a set of fixed
100 homozygous lines, descending from a F2 population. However, contrary to RILs, the parents
101 used to generate epiRIL population have identical (or almost identical) genomic sequence
102 but different DNA methylation profiles. EpiRILs are thus maximising epialleles segregation,
103 while reducing (if not removing completely) allelic segregation. EpiRILs can be used for
104 mapping epiQTLs.

105 QTL (Quantitative Trait Locus): A QTL is a locus of the genome at which genetic variation
106 correlates with variation of a quantitative trait.

107 EpiQTL (Epigenetic Quantitative Trait Locus): An epiQTL is a locus of the genome at which
108 variation in DNA methylation correlates with variation of a quantitative trait.

Additive alleles: Different alleles of a gene that combine in a way that the phenotype or expression level of the heterozygous is equal to the sum of each allele.

Dominant alleles: The dominant allele dictates the phenotype or expression level of the heterozygous, when paired with a recessive allele.

Transgressive transcripts: In the context of a hybrid, locus expression level that is not explained neither as additive, nor than as dominant allelic effect.

Definition and description of the epiRIL populations

The study of epiallele stability and phenotypic consequences can be performed by taking advantage of natural variation in *Arabidopsis thaliana*. DNA methylation at the level of genes has been shown to be highly polymorphic between *A. thaliana* accessions, making possible to follow epialleles segregation and their stability in F2 populations (Vaughn et al., 2007). Natural accessions not only differ in their levels of DNA methylation, but also in their genetic sequences, and genetic polymorphism can be used to identify the parent of origin for genomic regions in F1 and F2 populations (Greaves et al., 2012; Shen et al., 2012; Vaughn et al., 2007; X. Zhang, Shiu, Cal, & Borevitz, 2008).

However, the presence of genetic and epigenetic variation across *A. thaliana* natural accessions often impairs proper quantification of the epigenetic contribution to phenotypic differences. Indeed, several studies in plants (but also in mammals) reported many examples of DNA methylation variations associated to either local (*cis*) or distant (*trans*) changes in DNA sequence (Eichten et al., 2011; Gibbs et al., 2010; Hellman & Chess, 2010; D. Zhang et al., 2010). On the other hand, mutation rate of methylated cytosines is higher than

non methylated cytosines (Xia, Han, & Zhao, 2012), suggesting that DNA methylation and DNA sequence polymorphisms can be linked and also influence each others. Therefore, a classification of epialleles has been proposed depending on their link with DNA sequence polymorphism (Richards, 2006): (i) obligatory epialleles, for which a *cis* or *trans* genetic polymorphism influences the DNA methylation status; (ii) facilitated epialleles, which can be linked to or caused by a genetic polymorphism, but that are not fully dependent on it; and (iii) pure epialleles, that are not affected by any genetic changes.

Two epiRIL populations have been independently created in *Arabidopsis thaliana* (Figure 1), to maximise DNA methylation variation and minimise (if not abolishing) DNA sequence polymorphisms, in order to discriminate epialleles that are not influenced by DNA sequence polymorphisms (Johannes et al., 2008). These epiRILs have been generated by crossing an epigenetic mutant, *met1-3* (Reinders et al., 2009) or *ddm1-2* (Johannes et al., 2009), with its corresponding wild-type (Columbia-0 accession). The two parents thus have the same genome, except for the mutated gene, but they have very contrasting DNA methylation profiles. Each epiRIL within the population essentially contains a mosaic epigenome derived from either wild-type and *ddm1-2* or wild-type and *met1-3*.

Although both *met1-3* and *ddm1-2* mutants are hypomethylated, their effects on genome wide DNA methylation are different, and these differences are conserved in the epigenetic perturbations segregating in two epiRILs populations. The DNA methyltransferase MET1 maintains CG methylation in *Arabidopsis thaliana* and the *met1-3* null mutant is characterised by a virtual complete erasure of CG methylation and indirect loss of plant-specific non-CG methylation (Saze, Mittelsten Scheid, & Paszkowski, 2003). On the other hand, *DDM1* encodes an ATPase chromatin remodeler primarily involved in allowing DNA

156 methyltransferases to access heterochromatin (Zemach et al., 2013). *ddm1-2* mutation
157 mainly affects DNA methylation in all cytosine contexts (CG, CHG and CHH) at
158 heterochromatic TEs (Kakutani, Jeddeloh, & Richards, 1995; Lippman et al., 2004; Vongs,
159 Kakutani, Martienssen, & Richards, 1993). Consequently, the epialleles generated in *met1*-
160 derived epiRILs are equally distributed in euchromatic and heterochromatic areas, including
161 gene bodies that are exclusively CG methylated (Bewick et al., 2016; Catoni et al., 2017),
162 while epialleles in *ddm1*-derived epiRILs are mostly involving TE loci (Cortijo et al., 2014).
163 The *met1-3* mutant used to create the *met1*-epiRIL population also shows very severe
164 phenotypic defects, including reduced fertility (Mathieu, Reinders, Caikovski, Smathajitt, &
165 Paszkowski, 2007). Hence, a high level of mortality (29%) has also been observed while
166 propagating 100 individuals of the *met1*-epiRIL population over generations (Reinders et al.,
167 2009). On the contrary *ddm1*-derived epiRILs have been generated starting from the *ddm1*-
168 2 mutant, which displays only minor developmental defects. This strategy allowed the
169 production of a large population of 505 different *ddm1*-derived epiRILs, with no evidence of
170 selection against deleterious phenotypes (Colome-Tatche et al., 2012).
171 The crossing scheme of the two populations also differs. In both cases, the mutant (*met1-3*
172 or *ddm1-2*) has been crossed with a wild-type plant and only F2 plants segregating the wild-
173 type allele have been used to generate the epiRIL populations. The *met1*-epiRILs originate
174 directly from the F2 individuals resulting from this cross, while the *ddm1*-epiRILs descend
175 from a second back cross of the F1 with the wild-type. Thus, DNA methylation changes
176 segregate with a 1:1 ratio in the *met1*-epiRILs and with a 1:3 (mut/WT) ratio in the *ddm1*-
177 epiRILs.

178 [Insert Figure 1 here]

DNA methylation transgenerational stability and its phenotypic consequences

- Understanding of the stability of DNA methylation perturbations

Contrary to mammals, in plants there is no evidence of a consistent global resetting of DNA methylation during development, making the transmission of epialleles over generations more probable. Indeed, it has been shown that the loss of DNA methylation induced by the *ddm1-2* mutation can be stably inherited over many generations once the DDM1 wild-type allele is re-introduced (Kakutani, Munakata, Richards, & Hirochika, 1999). The analysis of the transmission of *ddm1-2* and *met1-3* induced hypomethylation at six TEs, after a cross with wild-type, showed that the hypomethylation is transmitted at some loci and reversed to a wild-type methylation state at other loci (Lippman et al., 2003). Methylated regions have been divided into two categories: (i) those that can form two distinct epialleles that are maintained over generations once in a WT background, and (ii) those that revert to the WT epigenetic state (remethylatable). EpiRILs are a great tool to study the mechanisms and consequences of DNA hypomethylation stability or reversion over generations. Indeed, the analysis of the DNA hypomethylation stability at multiple loci in three *ddm1*-epiRILs and three *met1*-epiRILs confirmed the presence of stable and remethylatable *ddm1*- and *met1*-induced hypomethylation (Reinders et al., 2009; Teixeira et al., 2009). Transgressive DNA methylation patterns have also been observed in these populations. Using bisulfite sequencing it was shown that remethylation to a level similar to wild-type was observed occurring at many loci in all cytosine contexts. This remethylation requires sRNA and factors involved in RNA-directed DNA methylation and is progressive over generations in the *ddm1*-epiRILs (Teixeira et al., 2009), while remethylation has been observed directly occurring in

the F1 in the case of *met1*-induced hypomethylation (Rigal et al., 2016). Further analysis of *cis* factors influencing remethylation in the *met1*-epiRILs as well as in the F2, containing the wild-type allele of MET1, originating from a backcross between *met1-3* and wild-type, showed that remethylation is associated with repetitiveness and relative scarcity of CpGs. In contrast, stable epialleles are associated with low copy number and high CpG content (Catoni et al., 2017). The link between these *cis* factors and the level of epigenetic stability was confirmed in rice (Catoni et al., 2017), and also observed generally associated to the susceptibility of transgenes to be epigenetically silenced (Sidorenko et al., 2017). This observation shows how epiRILs in *Arabidopsis thaliana* could be of great help to identify general rules associated to epiallele stability in different plant species or even for synthetic or heterologous DNA sequences (like transgenes).

- From epialleles to epigenomic recombination maps

The identification of epialleles in epiRILs has been used advantageously to identify the parental origin of genomic regions along the genome, exclusively using DNA methylation information (Colome-Tatche et al., 2012; Reinders et al., 2009). Parental origin was identified for three *met1*-epiRILs using whole-genome methylation analysis (Reinders et al., 2009). Genome-wide DNA methylation data for 123 *ddm1*-epiRILs were also used in order to construct a recombination map derived from 126 epialleles covering 81.9% of the total genome (Colome-Tatche et al., 2012). The genetic length of this map is comparable to those obtained from classical *Arabidopsis* crosses, suggesting that the hypomethylated loci segregating in the *ddm1*-epiRILs do not affect the global meiotic recombination rates. However, it has been seen on a local scale that recombination rates are reduced within

repeat-rich pericentromeric regions and increased in chromosome arms (Colome-Tatche et al., 2012). This remodelling of recombination hotspots, without changing the global rate, was also independently observed using *met1*-epiRILs (Mirouze et al., 2012). A later study shown that this remodelling of local recombination requires genes involved in the redistribution of interfering crossovers (Yelina et al., 2015). Interestingly, the creation of epigenomic recombination maps using epialleles has also been done using mutation accumulation (MA) lines in *Arabidopsis thaliana* (Hofmeister, Lee, Rohr, Hall, & Schmitz, 2017). MA lines are self-pollinated single-seed descent lines originating from a single founder, such that the lines are nearly genetically identical. MA lines display DNA methylation variation, and more than half of the differentially methylated regions identified in MA lines were stably transmitted in the progeny of a cross between two of them (Hofmeister et al., 2017). The creation of epigenomic recombination maps using stable DNA methylation variation is thus not restricted to epiRILs and will be of great interest to identify epialleles underlying phenotypic variation.

- Epialleles and phenotypic consequences

Knowing that a proportion of DNA methylation perturbations are transmitted through generations in *met1* and *ddm1*-epiRILs, one important question is to define if these can have phenotypic consequences. The two epiRIL populations have been extensively phenotyped for qualitative as well as quantitative traits such as flowering time, biomass or response to biotic and abiotic stresses. Two types of phenotypic variation have been observed. A first type of recessive variation has been observed sporadically occurring in only one epiRIL line and thus arose specifically during the creation of that line (Figure 2). These specific

phenotypic changes are unlikely to be transmitted from the parents used in the creation of the epiRIL populations, and it was shown that TE mobilisation impairing gene functions were the cause of such specific phenotypes in the *met1*-epiRIL population (Mirouze et al., 2009). The second type of phenotypic change is affecting a significant proportion of the epiRIL lines and is thus potentially inherited from the parents. We will discuss more in detail this second type of phenotypic change, as they are more likely to be caused by epialleles segregating in the epiRIL populations.

[Insert Figure 2 here]

One strong phenotype observed in the two epiRIL populations is delayed flowering time, which has been shown to be associated with the hypomethylated epiallele at the *FWA* locus (Johannes et al., 2009; Reinders et al., 2009). However, continuous variation for flowering time is still observed in the *ddm1*-epiRIL population after removing individuals for which late flowering is caused by this *fwa* epiallele (Johannes et al., 2009). This suggests that DNA methylation changes at other loci are also involved in the segregation of this trait in the *ddm1*-epiRIL population.

A large proportion of the *met1*-epiRIL population is also characterised with retarded growth (85% of *met1*-epiRILs) as well as delayed germination under elevated salinity (60% of *met1*-epiRILs). Moreover, 34% and 4% of *met1*-epiRILs showed respectively increased resistance or susceptibility to the biotrophic bacterial pathogen *Pseudomonas syringae* pv. tomato (Pst) (Reinders et al., 2009).

Given the high number of lines in the *ddm1*-epiRIL population (505 lines), many quantitative traits have been measured in this population and their heritability estimated (i.e. the degree of variation in the phenotypic trait in the population due to genetic, and here epigenetic, variation between individuals). A continuous variation and high heritability have been

276 observed for several traits such as flowering time, plant height, primary root length, fruit
277 number, total biomass and others (Cortijo et al., 2014; Johannes et al., 2009; Roux et al.,
278 2011). Many traits such as flowering time, plant height, fruit size, dry biomass and rosette
279 diameter have also been measured in common garden experiments, alongside natural
280 accessions of *Arabidopsis thaliana* (Roux et al., 2011). It was found that phenotypic
281 variation in the *ddm1*-epiRIL population displays a level of trait heritability similar to the
282 natural *Arabidopsis* accessions grown in parallel. Phenotypic plasticity, which is the ability of
283 one genotype to produce multiple phenotypes in response to the environment, has also
284 been measured for flowering time, plant height, fruit number, total biomass and root:shoot
285 ratio in response to drought and nutrient addition (Zhang et al., 2012). A high heritability
286 was observed for these traits in the absence and presence of environmental perturbations,
287 but also for their plasticity (Y. Y. Zhang, Fischer, Colot, & Bossdorf, 2013). Theoretical
288 predictions indicate that these heritability values are consistent with a small number of
289 parentally derived quantitative trait loci (QTL, see definitions). These results suggest that
290 phenotypic variability in the *ddm1*-epiRILs can be caused by the segregation of epialleles, or
291 by DNA sequence polymorphisms caused by mobilisation of transposable elements,
292 reactivated by DNA hypomethylation.

293 In order to identify the loci underlying heritable phenotypic variability in the *ddm1*-epiRIL
294 population, and to define their genetic or epigenetic origin, epigenetic quantitative trait loci
295 (epiQTL, see definitions) have been mapped in *ddm1*-epiRILs for flowering time and primary
296 root length (Cortijo et al., 2014). This was done taking advantage of a genetic map
297 generated using differentially methylated regions in 123 *ddm1*-epiRILs, and covering 81.9%
298 of the total genome (Colome-Tatche et al., 2012). Several epiQTLs were detected on
299 chromosomes 1, 4 and 5 for flowering time, and on chromosomes 1, 2 and 4 for primary

300 root length (Figure 3). These QTLs could be associated to epigenetic polymorphisms, but
301 also caused by TE mobilisation. In order to discriminate between these two possibilities,
302 association between DNA methylation status and primary root length was confirmed for the
303 markers at the peak of the three epiQTLs in an independent F3 population. Moreover, new
304 TE mobilisations detected at these epiQTLs in the epiRIL population are not present in this
305 F3 population. These results strongly suggest that changes in DNA methylation are causing
306 the epiQTLs detected for primary root length (Cortijo et al., 2014).

307 The next step will be to identify the epialleles underlying these epiQTLs. However, as for
308 mapping alleles underlying QTLs, this operation is challenging and will require more time
309 and work. A first step would be to generate a fine mapping population in order to reduce
310 the size of QTLs and thus the number of potential epialleles (Loudet, Gaudon, Trubuil, &
311 Daniel-Vedele, 2005). Once potential epialleles will be detected, manipulating their DNA
312 methylation status will be required in order to confirm the link between DNA methylation
313 and phenotypic variability at this locus. Targeted DNA methylation is still challenging but
314 could be now achieved using a deactivated Cas9 fused with a DNA methyltransferase (Vojta
315 et al., 2016), by VIGS (Bond & Baulcombe, 2015) or by using RNA hairpins to trigger RdDM
316 (Mette, Aufsatz, van der Winden, Matzke, & Matzke, 2000).

317 However, the complete characterization of epialleles responsible for the identified epiQTL
318 associated to traits of interest is not necessarily a requirement in order to use this
319 knowledge to improve plants. Methods such as marker-assisted selection could be used to
320 introgress the desired trait in the cultivar of interest, taking advantage of markers
321 associated to the identified epiQTL (Kumar et al., 2017). The DNA methylation status of
322 these markers, rather than the DNA sequence polymorphisms, would have to be used
323 during the selection process. Assays based on DNA digestion with enzymes sensitive to DNA

methylation, as for example McrBC (Teixeira et al., 2009), associated to qPCR, would provide a cheap and high throughput approach to perform such selection based on the markers epigenetic status.

[Insert Figure 3 here]

Using epiRILs to understand TE mobilisation

Transposable elements (TEs) are a heterogeneous group of mobile DNA elements integrated in the genome of virtually all organisms, with the ability to move from their original position to a new genomic location. TEs can be classified in two main classes based on their transposition strategy: (i) Class I TEs (or retrotransposons), which transpose with a copy-and-paste mechanism through reverse transcription of a RNA intermediate and (ii) Class II TEs, transposing with a cut-and-paste mechanism mediated by a transposase (Wicker et al., 2007). Although initially considered as selfish genes and assimilated to “junk DNA” (Doolittle & Sapienza, 1980), the importance of the contribution of TEs to gene and genome structure and evolution is currently recognised across the entire tree of life (Hurst & Werren, 2001; Rebollo, Romanish, & Mager, 2012), including plants (Lisch, 2013). Consequently, transcriptional silencing of TEs ensures genetic stability, and is controlled in plants by a network of self-reinforcing epigenetic pathways, marking TEs with repressive marks at the level of DNA (cytosine methylation) and chromatin (histone repressive marks). Therefore, epigenetics mutants often show release of TE expression, and have been used to reveal and study real time TE mobilization (Ito et al., 2011; A. Miura et al., 2001; Tsukahara et al., 2009). In this context, epiRILs represent a valuable alternative to homozygous *met1*, *ddm1* and other epigenetic mutants in studying TE mobilization for several reasons. First, epiRILs are in the wild-type genetic background and are therefore genetically and phenotypically

348 more stable compared to the mutant from which they derived (Reinders et al., 2009).

349 Moreover, the epiallele segregation and homozygous fixation that occurred through many

350 inbred generations contributed to “dilute” the epialleles with deleterious effects, reducing

351 the amount of developmental defects that are normally displayed in the homozygous

352 mutant. For example, the *Arabidopsis met1-3* mutation is semi-lethal with transgenerational

353 decrease of fitness, and homozygous mutant plants can be maintained viable for a

354 maximum of four generations (Mathieu et al., 2007). Although not as severe as for *met1-3*

355 mutants, *ddm1-1* and *ddm1-2* homozygous mutants accumulate strong phenotypic defects

356 through generations, (Kakutani et al., 1996). Stochastic bursts of several TEs independently

357 occur in different *ddm1* inbred lines, and are contributing to at least some of the

358 developmental phenotypes observed in *ddm1* (A. Miura et al., 2001; Tsukahara et al., 2009).

359 By contrast, *met1* and *ddm1*-derived epiRILs have been maintained for more than eight

360 generations without noticing a significant decrease in fertility (Johannes et al., 2009;

361 Reinders et al., 2009), providing a much more reliable platform to study transposition

362 events. Indeed, the mobilization of the Class II DNA transposon CACTA1 (Reinders et al.,

363 2009) and the Class I retrotransposon EVADE (EVD) (Mirouze et al., 2009) were reported in

364 *met1*-derived epiRILs, while not detected in the *met1-3* mutant. Similarly, many transposons

365 have been found active in *ddm1*-epiRILs, indicating that *ddm1-2* mutation is necessary to

366 release TE silencing, and that TEs can remain active after re-introduction of the DDM1 wild-

367 type allele (Cortijo et al., 2014; Gilly et al., 2014). In *ddm1*-epiRILs the fraction of the

368 demethylated genome was initially diluted through one *ddm1* backcross of the F1 with the

369 wild-type, reducing in average to 25% the fraction of hypomethylated genome inherited

370 from the *ddm1-2* mutant parent, and contributing to stabilize epiRILs phenotypes at late

371 generations.

Therefore, both *met1* and *ddm1* derived epiRIL populations demonstrated a longer transgenerational viability and stability compared to the mutant parents from which they are derived. The advantage of this condition is that the plethora of epiallelic effects and multiple TE activation observed in the homozygous mutants can be isolated in independent epiRILs, making it possible to study the activation and *de novo* silencing of independent TEs in real time experiments. For example, the transgenerational dynamic evolution of EVD mobilization was studied in inbred epiRILs (Mari-Ordonez et al., 2013). The EVD burst and its *de novo* silencing was reconstructed in a *met1*-epiRIL, observing that efficient silencing is associated to a change in small RNA composition, and consistently occurs approximately at the 14th generation after EVD activation, when its copy number in the genome reaches a threshold of 40 copies (Mari-Ordonez et al., 2013).

Although the first events of real time transposition were discovered in maize more than 50 years ago (Mc Clintock, 1950), the impact of TE mobilization on genome stability and the biology of complex organisms is still poorly investigated, and essentially extrapolated from comparative genomics and phylogenetic studies. This limitation is the direct consequence of the rarity of TE mobilization events so far observed in nature, likely due to the epigenetic silencing normally associated to repeated DNA sequences.

The most evident effect of TE mobilization is the recessive mutation of genes with a new TE insertion occurring in their coding region, in many cases producing a visible phenotype. Nonetheless, phylogenetic studies produced evidence of several TE-induced non-destructive effects on gene expression responsible for agricultural important traits in crops (Lisch, 2013). It is however unclear if these non-destructive effects derived from positively selected exceptional aberrant transposition events or are the result of transposition strategies of different TE families. In this scenario, epiRILs offer the opportunity to identify and

characterize new active TEs, and to study the impact of their real time mobilization across generations in a limited number of plant lines. Therefore, the study of epiRILs may contribute to elucidate the role of TE on genetic and biology in higher plants, and more generally in eukaryotic multicellular organisms.

Heterosis

Heterosis, or hybrid vigour, is a phenomenon describing the improved phenotype of a hybrid offspring compared to the average of both parents, first recorded by Charles Darwin in 1876 (Darwin, 1876). In agriculture, heterosis has been adopted as a routine strategy for plant breeding, leading to improved biomass, yield or resistance to biotic and abiotic stimuli in hybrids (Baranwal, Mikkilineni, Zehr, Tyagi, & Kapoor, 2012). Despite such an extensive use in agriculture, the underlying mechanisms of heterosis are still poorly understood. Traditionally, it is generally accepted that heterosis directly correlates with the level of genetic distance between the two parents (Birchler, Yao, Chudalayandi, Vaiman, & Veitia, 2010). However, more recent experiments performed in *Arabidopsis* have shown that hybrids generated from accessions with very similar genome can also display a high level of hybrid vigour (Groszmann, Greaves, Fujimoto, Peacock, & Dennis, 2013; Schneeberger et al., 2011), suggesting that epigenetic differences could also contribute to heterosis (Figure 4).

[Insert Figure 4 here]

Indeed, changes in small RNA level and DNA methylation have been associated to hybrid vigour in both interspecific (i.e. between species) or intraspecific (i.e. between accessions) hybrids systems studied in *Arabidopsis* (Greaves et al., 2012; Groszmann et al., 2011; Shen et al., 2012) and other plant species, including rice (Chodavarapu et al., 2012; G. He, He, & Deng, 2013), maize (Barber et al., 2012; G. He, Chen, et al., 2013), wheat (Kenan-Eichler et

420 al., 2011) and tomato (Shivaprasad, Dunn, Santos, Bassett, & Baulcombe, 2012). However,
421 the coexistence of genetic and epigenetic differences in hybrids makes it intrinsically
422 difficult to quantify the epigenetic contribution to heterosis.

423 In contrast, epiRILs are isogenic to wild-type but differ at localized hypomethylated
424 chromosomal areas. Interestingly, some lines from both *met1*-derived and *ddm1*-derived
425 epiRIL populations displayed increased biomass or higher resistance to a pathogen if
426 compared to wild-type Columbia-0 accession (Johannes et al., 2009; Reinders et al., 2009),
427 similar to what is observed in heterotic hybrids. These results suggest that epigenetic
428 variation by itself might be involved in the generation of hybrid vigour.

429 In a recent work, heterosis for growth-related traits was investigated in epigenetic hybrids
430 generated by pollinating *met1*-derived epiRIL plants with pollen from their isogenic wild-
431 type line (Col-0) (Dapp et al., 2015). In the case of one *met1*-derived epiRIL (epi31), a
432 consistent and reproducible increase in rosette size was observed in F1 plants compared to
433 both parental lines. Remarkably, epi31 displayed a clear parent-of-origin effect on hybrid
434 vigour, as also observed in certain crosses between *Arabidopsis* accessions (Barth, Busimi,
435 Friedrich Utz, & Melchinger, 2003; Meyer, Torjek, Becher, & Altmann, 2004). Although the
436 authors could not associate any change in gene expression with the hybrid vigour observed,
437 several additive, dominant and transgressive (see definitions) transcripts have been identify
438 in the F1 hybrids (Dapp et al., 2015), supporting the existence of multiple scenarios for DNA
439 methylation-mediated gene regulation in epi-hybrids.

440 More recently, the contribution of differences in parental methylation to heterosis was
441 quantified measuring six different traits in a larger panel of over 500 *A. thaliana* epi-hybrids
442 obtained starting from *ddm1*-derived epiRILs (Lauss et al., 2018). Several positive and
443 negative heterotic effects were documented, and specific differentially methylated regions

in parental genomes were associated with heterotic phenotypes observed in nineteen epi-hybrids (Lauss et al., 2018).

In conclusion, there is growing evidence supporting the epigenetic contribution to heterosis.

In this context, epiRILs may be the optimal tool to isolate and characterize epigenetic determinants of hybrid vigour, for example by mapping epiQTLs associated to different favourable traits. In addition, altering the epigenetic landscape of parents can potentially increase the heterotic effect of hybrids, and could be used as a tool to increase plant productivity.

Challenges with crops

The investigation of the epigenetic landscape in Arabidopsis epiRILs critically contributed to reveal general plant epigenetic properties and mechanisms. Such findings include the mapping of epiQTLs (Cortijo et al., 2014), the discovery of genetic properties that predict epialleles, common in Arabidopsis and rice (Catoni et al., 2017), and a model for origin and evolutionary consequences of gene body DNA methylation in Angiosperms (Bewick et al., 2016). However, despite a general conservation of most epigenetic factors and properties across plants, epiRILs are so far only available for *Arabidopsis thaliana*. Creating epiRILs in crops could improve our understanding of the source of epiallelic creation and also help detecting epialleles with potential agronomic advantages.

The introduction in crops of a level of epigenetic variation similar to that observed in Arabidopsis epiRILs might be of great interest for agriculture. Especially when considering that crops have larger genomes containing a much higher number of transposons and repetitive DNA, suggesting an elevated potential for the generation of epialleles.

Consistently, rice, maize and tomato mutants in components of epigenetic regulation

display strong developmental phenotypes and partial or complete infertility (Gouil & Baulcombe, 2016; Hu et al., 2014; Li et al., 2014). Remarkably, developmental phenotypes described in crop epigenetic mutants do not correlate with extensive genome hypomethylation as observed in *Arabidopsis* (Mathieu et al., 2007), suggesting that in most plants small perturbations of the methylome have stronger deleterious phenotypic effects than in *Arabidopsis*.

Taking this into account, the generation of crop epiRILs may be impaired by the inability of producing viable hypomethylated mutants required for the initial cross. However, alternative strategies should be considered to induce stable epiallele formation without affecting plant viability (Figure 5).

[Insert Figure 5 here]

One possibility to reduce genome methylation is the use of hypomethylated partial loss-of-function epigenetic mutants with mitigated deleterious developmental phenotypes. In *Arabidopsis*, while the null *met1-3* allele causes complete loss of CpG methylation and is semi-lethal (Mathieu et al., 2007), the partially functional MET1 protein produced in the *met1-1* allele can retain CpG methylation in approximately one quarter of the genome, causing only minor developmental defects and allowing transgenerational conservation of the *met1-1* mutation in the homozygous form (Kankel et al., 2003). In addition, mobilization of TEs has also been observed in the *met1-1* mutant background (Griffiths, Catoni, Iwasaki, & Paszkowski, 2018) as well as the formation of epialleles that are stably maintained for several generations after transgenic complementation with a wild-type MET1 allele (Catoni et al., 2017). This suggests that the use of partial loss-of-function mutants might replace null alleles in epiRIL construction, if a viable knock-out mutant cannot be obtained. However, the production of partial-loss of function mutants for a chosen gene may be difficult to achieve

492 in plants, and is normally associated to fortuitous screening starting from random
493 mutagenized populations. Nonetheless, DNA editing strategies, such as CRISPR/ CAS9 (Cong
494 et al., 2013) and TALEN (Miller et al., 2011) have been successfully extended to plants,
495 allowing an unprecedented high level of accuracy in targeting chromosomal sequences to
496 induce mutations (Malzahn, Lowder, & Qi, 2017). Using these approaches, the effect of well
497 know partial loss-of-function mutations observed in Arabidopsis might be more easily
498 obtained in the species of interest by targeting a similar mutation in the corresponding
499 homologous genes.

500 Alternatively, passive DNA hypomethylation has been proposed to occur during
501 gametogenesis in heterozygous *met1* mutant. The haploid male and female gametophytes
502 undergo two and three post-meiotic divisions, respectively. Therefore, genomic DNA is
503 duplicated in gametophytes with the *met1* mutant allele, in absence of the MET1
504 methylation maintenance system, leading to the passive reduction to 50% and 75% of the
505 genome methylation respectively in male and female gametes (Saze et al., 2003). This
506 hypothesis was confirmed by later studies, observing also a genome-wide demethylation
507 and the formation of stable epialleles in heterozygous inbred *met1* mutant lines, similar to
508 what was observed in epiRILs (Catoni et al., 2017; Stroud, Greenberg, Feng, Bernatavichute,
509 & Jacobsen, 2013). Therefore, genome-wide hypomethylation in crop plants may be simply
510 achieved by inbreeding the usually more fertile heterozygous *met1* mutant, without the
511 necessity of a viable homozygous mutant allele.

512 One alternative to the generation of epigenetic mutants is the use of drugs interfering with
513 epigenetic pathways. Inhibitors of DNA methylases, such as 5-Azacytidine and Zebularine,
514 have been successfully used to induce DNA demethylation in plants (Griffin, Niederhuth, &
515 Schmitz, 2016; Pecinka & Liu, 2014), including crops (Sano, Kamada, Youssefian, Katsumi, &

516 Wabiko, 1990; Santos et al., 2002; Zhu et al., 2018). Although most of hypomethylation and
517 transcriptional changes induced by these drugs are only transient (Baubec, Pecinka, Rozhon,
518 & Mittelsten Scheid, 2009), transgenerational effects have been observed in rice treated
519 with 5-Azacytidine (Sano et al., 1990). Recently, simultaneous application of Zebularine and
520 the RNA polymerase II inhibitor α -amanitin on *Arabidopsis* wild-type seedlings was sufficient
521 to mobilize the heat-responsive Class I retrotransposon ONSEN, demonstrating that drug
522 application can efficiently release transposon transcriptional silencing (Thieme et al., 2017).
523 Finally, another very valuable alternative in order to reduce DNA methylation in plant is the
524 heterologous expression of enzymes promoting DNA hypomethylation. For example, the
525 human Ten-eleven translocation (TET) methylcytosine dioxygenases are an enzyme family
526 catalysing the conversion of 5mC in 5-hydroxymethylcytosine (5hmC), and are involved in
527 active DNA demethylation in embryonic stem cells (Tahiliani et al., 2009). The transgenic
528 expression of TET3 catalytic subunit in *Arabidopsis* was enough to decrease DNA
529 methylation at ribosomal repeats (Hollwey, Watson, & Meyer, 2016). In addition, the
530 transgenic expression of the same TET3 gene in Tomato induced hypomethylation and
531 ectopic expression of the CEN1.1 gene in leaves, promoting vegetative growth (Hollwey,
532 Out, Watson, Heidmann, & Meyer, 2017). In a more recent work, ectopic overexpression of
533 a different TET gene in *Arabidopsis* induced widespread DNA demethylation and phenotypic
534 variations, mimicking the effects of *met1* mutation (Ji et al., 2018). In addition, a Cas9-based
535 targeted demethylation system using the TET1 catalytic subunit was recently generated and
536 was shown to be able to target demethylation and activate gene expression when directed
537 to known switchable epialleles in *Arabidopsis* (Gallego-Bartolomé et al., 2018).

The combination of these approaches could thus potentially be used in order to promote global or specific changes in DNA methylation profiles and be the first step to create epiRILs in crops.

Conclusion

Arabidopsis epiRIL populations have allowed major advances in understanding the genetic determinant controlling DNA methylation stability as well as mechanisms involved in the transgenerational transmission of epigenetic information. Several studies used epiRILs to highlight the phenotypic consequences of epiallele segregation and the epigenetic contribution to quantitative traits. While epiRILs have been initially created with the intention of minimising DNA polymorphisms, the TE reactivation induced by the global loss of DNA methylation has been used advantageously in order to better understand how TE mobilisation is controlled, and to study the transgenerational effect of TE activation. EpiRILs have also helped to better understand the importance of DNA methylation on heterosis, commonly used in crops to improve yield.

The next step to extend the epigenetic potential to improve agricultural traits will be the creation of epiRILs in crops. This step is challenged by the amount of developmental defects associated to genome wide hypomethylation observed in epigenetic mutants. Nonetheless, the better understanding of the epigenetic contribution to phenotypes, and the use of more sophisticated genome editing strategies might be critical to successfully obtain crop epiRILs in the near future.

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874

Figure legends:

Table 1: Non-exhaustive list of known epialleles in plants.

Figure 1: Allelic and epiallelic segregation in RIL and epiRIL populations.

RIL populations (left) are usually created by crossing two distinct *Arabidopsis* accessions that are different in their genomes (depicted with different chromosome colours) and epigenomes (depicted as full or empty dots beside chromosomes). Alleles and epialleles are thus segregating in F2 population derived by this cross, and fixed in homozygous form by self-pollination and single seed-descend. By contrast, epiRILs (right) are created by crossing parents that have identical (or almost identical) genomic sequence but different DNA methylation profiles. This is obtained in *Arabidopsis* by mutation of *MET1* or *DDM1* genes (represented by a red horizontal line on chromosome sequence), coding for factors involved in DNA methylation maintenance. During the generation of epiRILs, only F2 plants with a *MET1* or *DDM1* wild-type allele are carried out, to avoid new events of genome wide hypomethylation. EpiRILs are thus maximising epialleles segregation, while reducing (if not removing completely) allelic segregation.

Figure 2: Origin of phenotypic changes observed in epiRILs.

Phenotypic changes occurring in epiRILs are of two types. The first type (left) is sporadic and recessive and occurring specifically in one line, probably caused by TE mobilisation or other genetic mutation. These phenotypes are unlikely to be transmitted from the parents used in the creation of the epiRIL populations. The second type of phenotypic changes (right) appears on a significant proportion of epiRIL lines. These traits are potentially inherited from the parents and likely caused by epialleles segregating in the epiRIL populations.

899

900 Figure 3: Principle of epiQTL mapping in epiRILs for root length, followed by epiallele
901 identification and validation.

902 In order to identify epiQTLs for a quantitative trait, every line of the population is
903 phenotyped (top left) and epigenotyped (top right). EpiQTLs are then identified by
904 measuring the co-segregation of phenotype and epigenotype. Several QTLs were identified
905 on chromosomes 1, 2 and 4 for root length in the *ddm1*-derived epiRILs (middle). The next
906 step is to identify epialleles underlying epiQTLs and to validate them by changing their DNA
907 methylation level (bottom).

908

909 Figure 4: Comparison of epi-hybrid and intraspecies hybrid in *Arabidopsis thaliana*.

910 Examples on enhanced vigour in an epi-hybrid, compared with its two parents, epi31 and
911 wild-type Col-0 (top), and in an intraspecies hybrid compared to its two parent accessions,
912 Col-0 and C24 (bottom). In both cases, the epi-hybrid and the intraspecies hybrid are bigger
913 than their parents, indicating a heterotic effect.

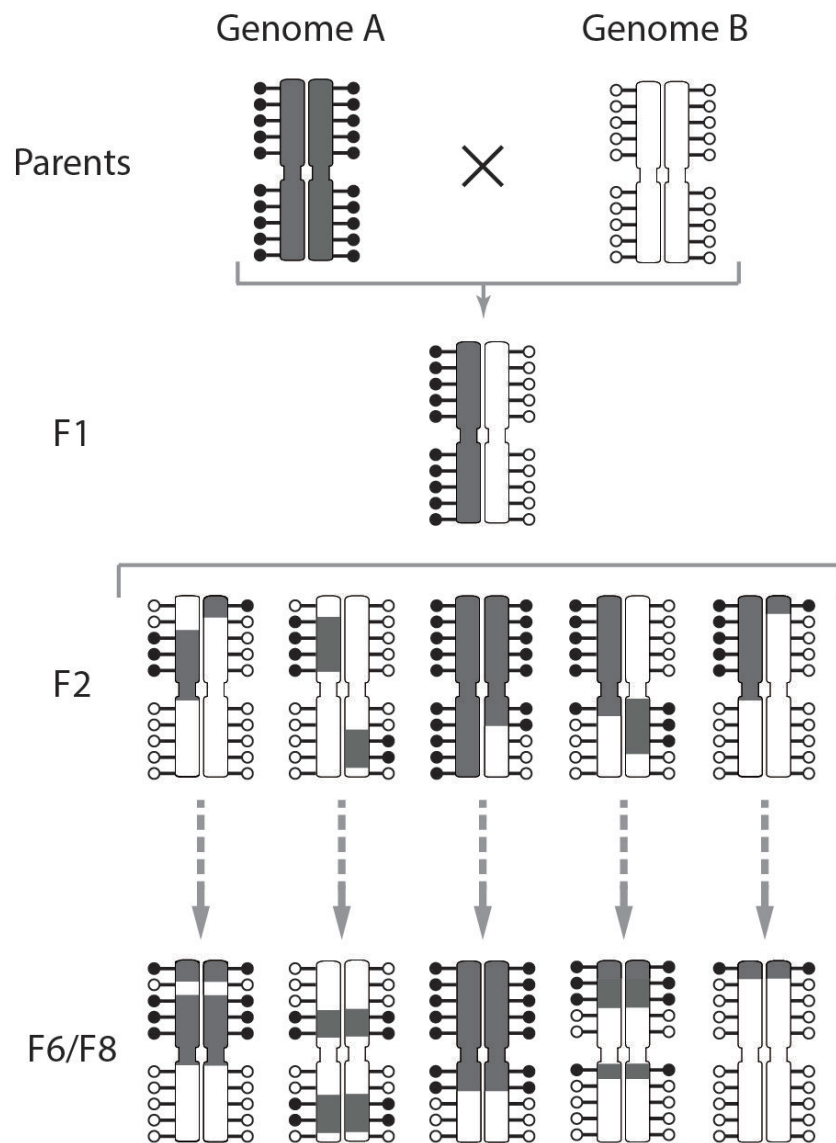
914

915 Figure 5: Different approaches to induce global DNA demethylation in order to create epiRIL
916 populations.

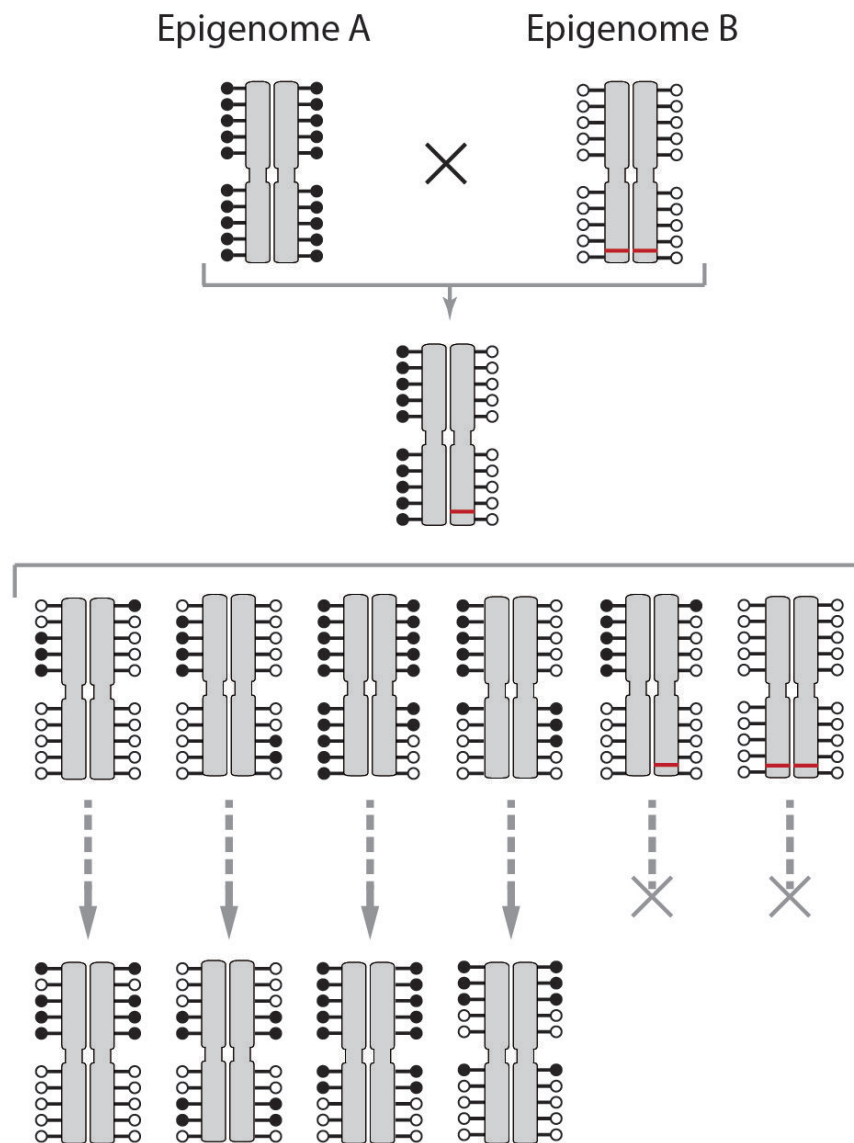
917 In wild-type, DNA maintenance mechanisms ensure conservation of epigenetic marks (i.e.
918 DNA methylation, represented as black dots). In *met1* or *ddm1* knock-out mutants, DNA
919 methylation is strongly impaired and normally associated to strong developmental
920 phenotype. Alternative strategies to reduce DNA methylation limiting the impact on plant
921 fitness include the use of partial loss-of-function mutations with partial de-methylation; the
922 self-propagation of heterozygous knock-out mutants, resulting in gametophyte

923 hypomethylation; the application of drugs interfering with methyltransferase activity; and
924 the ectopic overexpression of TET methylcytosine dioxygenases.
925

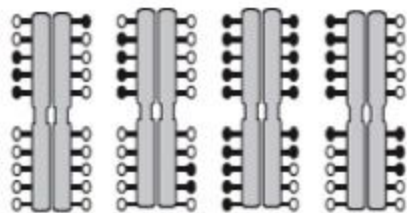
RIL generation



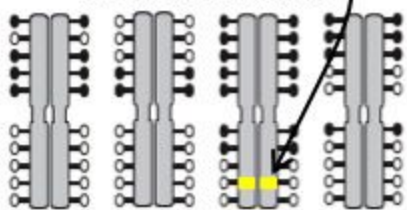
epiRIL generation



Phenotypic changes
caused by TE mobilisation

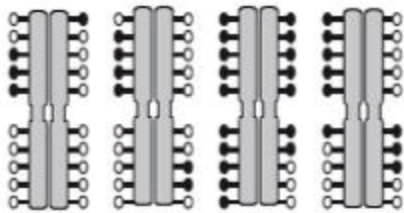


TE mobilization

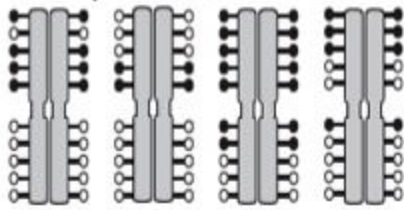


Sporadic phenotype

Phenotypic changes
caused by epiallele



Epialleles fixation



Segregating phenotype

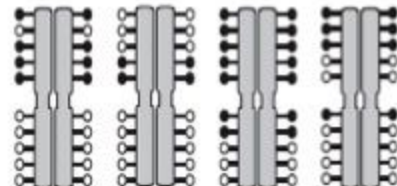
Phenotype

Line 1 Line 2 Line 3 Line 4...

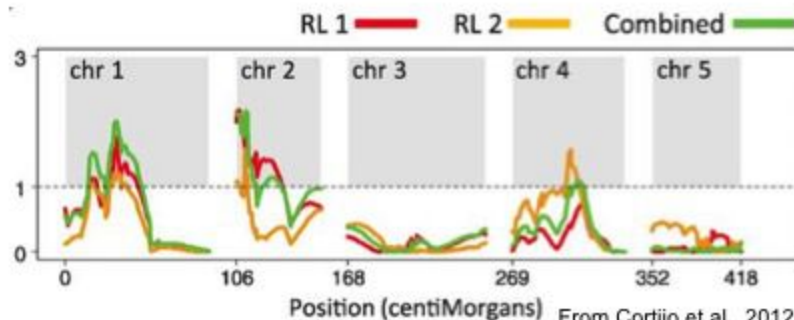


Epigenotype

Line 1 Line 2 Line 3 Line 4...

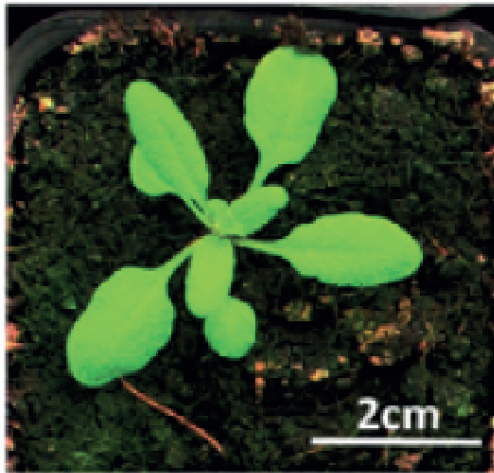


epiQTL mapping



Epiallele identification and validation

WT (Col-0)



epi31 x WT

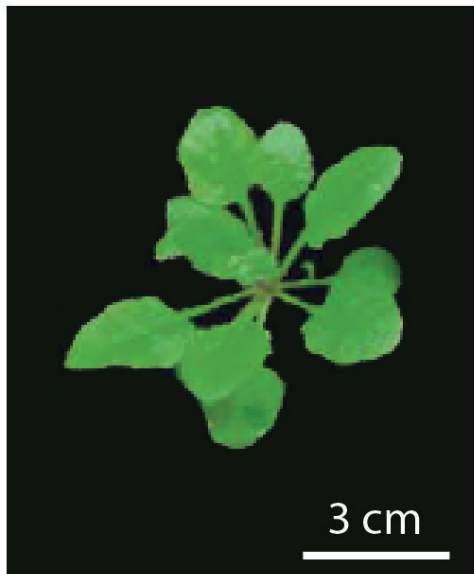


epi31 (Col-0)



From Dapp et al. 2015

Col-0



Col-0 x C24

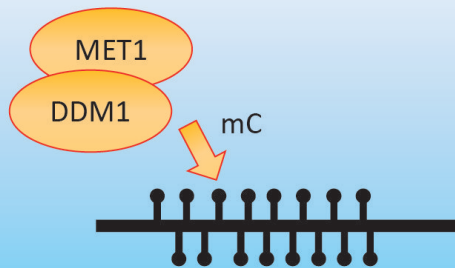


C24

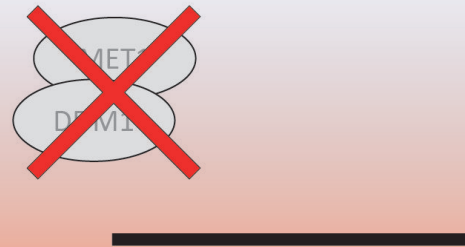


From Chen 2010

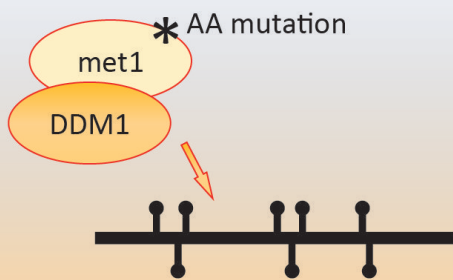
Wild type



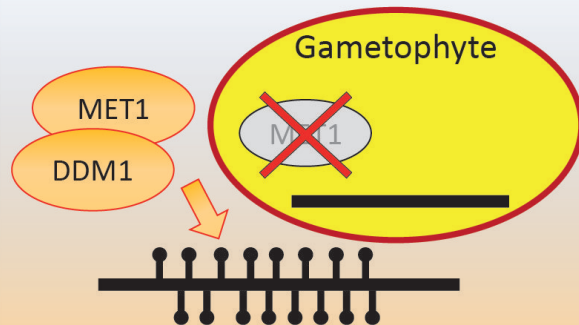
Knock-out mutants



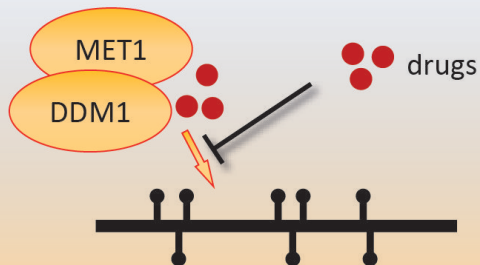
Partial loss-of-function mutants



Gametophytic de-methylation



Methyltransferase inhibitors



Heterologous enzyme expression

